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Production and Characterization of Monoclonal Antibodies Specific for a Glycosylated Polypeptide of Human Cytomegalovirus

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Nine hybrid cell lines producing antibodies specific for cytomegalovirus (CMV) antigen were obtained after fusion of P3/X63-Ag8 myeloma cells with spleen cells from BALB/c mice immunized with CMV complement-fixing antigen. By the immunoblot technique, five of nine antibodies (4D11, 7B4, 7D2, 8E3, and 8E10) were identified as being reactive to a CMV glycosylated polypeptide with molecular weight of 66,000 (GP66). Four other antibodies (1B8, 8E9, 4D2, and 7E2) appeared to be reactive with CMV antigen(s) only if the antigen was not denatured by sodium dodecyl sulfate. These remain unassigned until further studies are done. With the enzyme-linked immunosorbent assay (ELISA), competitive bindings were performed with a constant amount of horseradish peroxidase-conjugated antibody and various concentrations of unconjugated homologous and heterologous antibodies on CMV antigen-coated ELISA wells, and the antigenic determinant specific for each antibody was determined. The nine antibodies could be classified into six different groups, each group reacting with a different epitope or a different region with two or more antigenic determinants which are so close to each other that they cause binding inhibition. They are groups A (4D11), B (7B4, 8E10), C (7D2), D (4D2, 7E2, 8E9), E (8E3), and F (1B8). The extent of competition among antibodies within each group was the same. By using the two antibodies that reacted with different epitopes on GP66, a double-antibody sandwich ELISA method was developed. The method was sensitive enough to detect as little as 50% of the antigen present in one infected cell or 0.000245 U of CMV complement-fixing antigen per test well. Other strains of CMV (David, Kerr, Espilat, C-87, and five clinical isolates) gave positive results, whereas herpes simplex virus types 1 and 2, varicella-zoster virus and Epstein-Barr virus nuclear antigen preparations did not. By the indirect immunofluorescence assay, antibodies 4D11 and 8E3 were able to detect GP66 in the nucleus of CMV-infected F-5000 human embryonic fibroblasts as early as 2 h postinfection and were superior in this respect to the remaining seven antibodies tested. By the double-antibody sandwich ELISA, the presence of GP66 in CMV-infected cells was detected as early as 2 h postinfection.

Human cytomegalovirus (CMV) has been implicated in various disease syndromes in humans. Fetal CMV infection is a major cause of birth defects and second only to Down's syndrome as a cause of congenital mental retardation (1, 2, 10–14, 22, 23, 27). Although postnatal CMV infection in the normal host is generally benign, CMV infection is a serious medical problem in patients undergoing organ transplantation (3). Recently, a possible role for CMV in acquired immune deficiency syndrome has been suggested (6, 7). CMV has been shown to be transmitted by blood transfusions (18). Because of the scarcity of specific antisera to CMV, diagnosis of CMV infections by small diagnostic laboratories often relies on the recognition of a specific type of viral cytopathic effect which develops slowly (days to weeks) in suitable human embryonic fibroblast cells inoculated with clinical specimens. There is a need to develop a rapid and specific diagnostic test for the detection of CMV in blood, sera, urine, and other clinical specimens from the patient. Such a test would also prove useful in screening donors' blood for CMV before transfusion. One of the most objective, sensitive, and simple immunological methods for the detection and quantitation of viral antigens is the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA), but this would require a highly specific antisera to CMV antigen. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic

analysis of purified CMV (14) and purified dense bodies (20), which are usually present in the infected cells, indicated that a glycosylated polypeptide with a molecular weight of 66,000 (GP66) represents about 20 and 35% of the total protein present, respectively. In addition, a glycosylated polypeptide with the same molecular weight is present as a major glycosylated polypeptide in complement-fixing (CF) antigens prepared from different strains of CMV (13). Therefore, development of a double-antibody sandwich ELISA for the detection of GP66 might provide a highly sensitive and rapid diagnostic method. With this goal in mind, we developed monoclonal antibodies which reacted with different antigenic determinants on GP66 of CMV (strain AD169) and devised a double-antibody sandwich ELISA for detection of GP66.

MATERIALS AND METHODS

Cells. Human embryonic fibroblasts, F-5000, were from Flow Laboratories, Rockville, Md. The growth medium was Earle minimal essential medium containing 10% fetal bovine serum and 50 μg of gentamicin per ml.

Viruses. The AD169 strain of CMV was obtained from Adrian Chappell of the Centers for Disease Control, Atlanta, Ga. The Davis, Espilat, and Kerr strains of CMV were provided by Joseph Waner of the University of Oklahoma, Oklahoma City. The other strains of CMV used in the study were Towne (obtained from Stanley Plotkin, The Wistar Institute, Philadelphia, Pa.), C-87 (obtained from M. Benyesh-Melnick, Baylor University, Houston, Tex.), G-35 (obtained from Joseph Pagano of the University of North Carolina, Chapel Hill), and five clinical CMV isolates (A106, A148, A164, H. D., and D. H.) from our viral diagnostic laboratory. The McIntyre strain of herpes simplex virus type 1 (HSV-1), the Curtis strain of HSV type 2 (HSV-2), and the Ellen strain of varicella-zoster virus obtained from the American Type Culture Collection, Rockville, Md., were used. Epstein-Barr virus nuclear antigen prepared from Raji cells was provided by Helen Warner of our institute. The Epstein-Barr nuclear antigen preparation contained 4 U of antigen as determined by CF test.

CF test. The micro-CF method described by Waner et al. (27, 28) was used. The endpoint was the highest dilution that gave 75% (3+) fixation, which was determined by the amount of sheep erythrocytes sedimented in the presence of a constant amount of human reference serum to human CMV obtained from Flow Laboratories.

Preparations of CMV antigens and purified CMV. The methods described by Kim et al. (13, 14) were used for preparation of CMV antigens and purified CMV. Briefly, F-5000 cells infected with virus at a multiplicity of infection of about 5 PFU were scraped from the surface of 32-oz (946-ml) Brockway bottles with a rubber policeman and washed three times with phosphate-buffered saline (PBS) when they exhibited 100% cytopathic effect at 6 days postinfection (p.i.). Cells were resuspended in 5 ml of 0.1 M glycine-buffered saline (pH 9), sonicated for 1 min with a Branson Sonifier (model 140 equipped with a microtip; Heat Systems Ultrasonics, Plainview, N.Y.), and held overnight at 4°C. The material was centrifuged at 2,000 × *g* for 10 min, and the supernatant fluid was used as the CF antigen preparation. Control antigen was prepared from uninfected F-5000 cells in a similar way.

Extracellular fluid from CMV-infected cultures (6 days p.i. exhibiting 100% cytopathic effect) was pooled and centrifuged at 2,000 × *g* for 10 min in a Sorval GSA rotor to remove cellular debris. The supernatant fluid was removed and centrifuged at 130,000 × *g* for 1 h in a Beckman Ti45 rotor. The pellet was homogenized with a Dounce homogenizer and centrifuged at 2,000 × *g* for 10 min. The supernatant fluid was put on a column (2.5 by 85 cm) of Bio-Rad gel A-15m, equilibrated with PBS (pH 7.4). Most of the virus was recovered in the void volume, and this was then layered onto 30 ml of a 20 to 50% potassium tartrate gradient (Beckman SW27 rotor tubes), which was centrifuged at 113,000 × *g* for 3 h. A visible band which formed at a density of between 1.20 and 1.21 g/cm^3 was collected and dialyzed against PBS buffer for 2 h. The dialyzed material was put on a second tartrate gradient and centrifuged for 18 h at 113,000 × *g*. The virus band at a density between 1.20 and 1.21 g/cm^2 was collected and used as purified virus after dialysis against PBS buffer.

Preparation of hybrid cell lines. Procedures for producing antibody-secreting hybrid cell lines described by Tagoshi et al. (25) were modified and used. Hybrid cell lines producing antibodies to CMV antigen were obtained after fusion of P3/X63-Ag8 myeloma cells (obtained from James Chen, University of Texas, M.D. Anderson Hospital, Houston) with immune spleen cells. BALB/c female mice (from Jackson Laboratories, Bar Harbor, Maine) were immunized intraperitoneally with 100 μg of CMV CF antigen (strain AD169) per ml mixed with complete Freund adjuvant. At 7, 14, and 21 days after the initial injection, mice were immunized intraperitoneally with 100 μg of the antigen mixed with incomplete Freund adjuvant. Seven weeks after the last injection, the mice were given an intravenous booster of antigen without adjuvant. The spleen cells were used 3 days later for fusion, at a ratio of spleen cells/myeloma cells of 5:1. Polyethylene glycol 1000 (Fisher Scientific Co., Fair Lawn, N.J.) was used as the fusing agent.

The cells were maintained in Iscoves modified Dulbecco minimum essential medium containing 15% fetal bovine serum, 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, and 4.0×10^{-7} M aminopterin for 14 days. Subsequent feedings with medium containing only hypoxanthine and thymidine were performed on days 18, 22, and 25. After day 25 cells were fed with medium without hypoxanthine, thymidine, and aminopterin.

Hybrid cells were tested for CMV antibodies by the ELISA technique, using CMV antigen-coated Dynatech 96-well ELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.). Hybrids that produced CMV antibody were cloned three times by limiting dilutions, using normal BALB/c mouse macrophages (2×10^4 cells per well) as a feeder layer. Subsequently, hybrids were grown in tissue culture flasks. Cloned hybrid cell lines that continued to produce antibody for 3 months

were grown, and 10^7 cells were used for intraperitoneal injection of BALB/c mice which had been pretreated with 0.25 ml of pristane 1 and 8 days before injection. Ascites fluid obtained from the inoculated mice was used as a source of high concentrations of monoclonal antibodies. Ascites fluids collected from mice were pooled and the pool was diluted fourfold with PBS. An equal volume of saturated ammonium sulfate was added at 4°C, and the precipitated protein was collected after centrifugation for 30 min in a Sorvall SS-34 rotor at 12,000 × *g*. The precipitate was dissolved in PBS and dialyzed against 0.01 M Tris-hydrochloride buffer, pH 8. The supernatant fluid from the original dialysate was absorbed to a DE52 DEAE-D-cellulose (Whatman, London, England) column equilibrated with 0.01 M Tris-saline (pH 8) and eluted with a linear NaCl gradient (0.05 to 0.15 M NaCl in 0.01 M Tris-saline, pH 8). Monoclonal antibodies obtained in this manner are referred to herein as purified antibody.

ELISA test for detection of CMV-specific antibodies. To distinguish between hybrids producing CMV-specific antibody and normal F-5000 cell-specific antibody, an ELISA screening test was performed. Dynatech Immulon plates with 96 wells coated with CMV antigen and normal cell antigen were prepared. The antigen coating was accomplished by adding 50 μl of CMV antigen in carbonate buffer, pH 9.6, and incubating for 2 h at 37°C. Control F-5000 cell antigen-coated plates were prepared in the same manner. After the wells were washed with PBS six times, 50-μl amounts of culture fluid were added to both CMV antigen-coated wells and normal F-5000 cell antigen-coated wells; the plates were then incubated for 2 h at 37°C. At the end of incubation, the wells were washed six times with PBS and incubated for a further 2 h at 37°C with goat anti-mouse immunoglobulin G (IgG) and IgM antibody conjugated with horseradish peroxidase (HRP) (Tago Inc., Burlingdale, Calif.). After the wells were washed six times with PBS containing 1% Triton X-100, *O*-phenylenediamine (OPD) substrate solution (50 mg of OPD, 20 μl of 30% H_2O_2 in 100 ml of citrate buffer, pH 5) was added and the plates were incubated for 30 min at room temperature (RT). The color change was measured with a Dynatech Microelisa Auto Reader MC640, using a 490-nm filter. Only clones secreting antibody which reacted with CMV antigen-coated wells and not with normal F-5000 cell antigen-coated wells were selected for further characterization.

Gel electrophoresis. Electrophoresis in 0.1% SDS-containing polyacrylamide gels was performed as described by Kim et al. (13, 14) except that 9% polyacrylamide was used as the separating gel topped with a 3% stacking gel.

Molecular weight determination. The method described by Weber and Osborn (29) was used to estimate the approximate weights of viral protein by gel electrophoresis. The markers were as follows: myosin (200,000), β-galactosidase (130,000), phosphorylase (92,500), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Determination of protein. Protein concentrations were determined by the protein-Coomassie brilliant blue binding assay, using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Immunochemical staining of SDS-gel blots. CMV-specific monoclonal antibodies were analyzed by immunochemical staining of separated CMV proteins from SDS-gels which were blotted onto a nitrocellulose sheet by transverse electrophoresis at 10 V/cm for 30 min, using a Hoeffer transfer apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.) as described by Towbin et al. (26) and Burnette (5). In each experiment, several blots of the same combination of samples, run in the same slab, were made; one blot from each set was stained for protein with 0.1% amido black in methanol-acetic acid-water (4.5:1:4.5) for 3 min, washed in water for 4 min, and destained in methanol-acetic acid-water (90:2:8). Once the reliability of the transfer had been established, other blots were stained with monoclonal antibodies.

The blots were incubated in 0.01 M Tris buffer (pH 7.4)–0.2 M NaCl–3% bovine serum albumin for 1 h at 37°C on a rocking platform. The blots were transferred to a fresh solution of Tris-saline containing 1:1,000 dilutions of purified monoclonal antibody from ascites fluid. After incubation for 2 h at RT, the blots were washed with four changes of Tris-saline for 30 min. They were next incubated with a 1:1,000 dilution of HRP-conjugated goat anti-mouse IgG antibody (Tago Inc.) in Tris-saline plus 3% bovine serum albumin. After 1-h incubation at RT, the blots were washed four times with Tris-saline and then immersed for 5 to 10 min in 0.048% 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.)–0.1% H_2O_2 in Tris-saline. The blots were washed in distilled water, dried, and kept in the dark. Positive antibody-antigen reactions were evidenced by development of a blue color due to the binding of goat anti-mouse IgG HRP conjugate to monoclonal antibody, which was bound to the specific polypeptide band.

Indirect immunofluorescence assay (IFA). Virus-infected and control F-5000 cells were prepared in Lab-Tek tissue culture chamber slides (Miles Laboratories Inc., Naperville, Ill.). Cells were infected with cell-free virus at a multiplicity of infection of 10 PFU, and at different times p.i. the infected cells on slides were fixed in cold acetone (−20°C) for 10 min and air dried. To each well of the slide 40 μl of a 200-μg/ml amount of purified monoclonal antibody from ascites fluid was added and incubated for 30 min at 37°C in a humidified chamber. The slides were washed three times in PBS for 30 min at RT. Each well was then reacted with 40 μl of an optimum solution (1:10) of fluorescein-conjugated goat anti-mouse IgG (Tago Inc.) for 30 min at 37°C in a humidified chamber. The slides were washed for 30 min in three changes of PBS. Cover slips were mounted with glycerol-PBS, pH 7.4 (1:1), mounting fluid. The slides were examined with a Zeiss fluorescent microscope.

Virus neutralization test. Purified monoclonal antibody was diluted in minimal essential medium with 1% fetal bovine serum to contain antibody at concentrations of 100, 10, and 1 μg/ml. Equal volumes of serum dilutions and cell-free CMV, strain AD169, estimated to contain about 50 to 200 PFU, were mixed, incubated at 37°C for 1 h, and inoculated onto F-5000 monolayers to determine PFU. The plaque assay method described by Wentworth and French (30) was used.

Characterization of antibody subclasses. Monoclonal antibody secreted in culture fluid was characterized by Ouchterlony double-diffusion tests with goat anti-mouse antibody specific to IgG1, IgG2a, IgG2b, IgG3,

and IgM. The reagents were obtained from Meloy Laboratories Inc., Springfield, Va.

Preparation of HRP-conjugated monoclonal antibody. Purified monoclonal antibody from ascites fluid was conjugated with HRP (Sigma) by a modification of the two-step sodium periodate oxidation-sodium borohydride reduction procedure described by Wilson and Nakane (31).

Double-antibody sandwich ELISA for detection of CMV antigen. Dynatech Immunolon plates with 96 wells were coated with 50 μl of purified monoclonal antibody from ascites fluid (5 μg/ml in carbonate buffer, pH 9.6) for 2 h at 37°C. After the plates were washed for 20 min with three changes of PBS–1% Triton X-100, 50 μl of CMV (strain AD169)-infected cells solubilized with Triton X-100 (1% final concentration) for 30 min at 37°C and diluted in PBS–1% Triton X-100 was added and incubated for 2 h at RT. After the plates were washed, the optimum dilution of HRP-conjugated monoclonal antibody was added and plates were incubated overnight at RT. The plates were then washed for 20 min, and OPD substrate solution was added. After 30 min at RT, 75 μl of 1 M H_2SO_4 was added and the color change was determined with a Dynatech Microelisa Auto Reader MC640 with a 490-nm filter. For a negative control, normal F-5000 cells solubilized in Triton X-100 (1% final concentration) was used. The cutoff point between positive and negative was the average optical density (OD) value of the negative controls (quadruplicates) plus 2 standard deviations. Positive results were defined as the highest dilution of a viral antigen giving an OD value higher than the cutoff OD value.

Determination of antigenic determinants specific for each monoclonal antibody by competitive binding assay. Using the ELISA technique, competitive binding assays were performed with HRP-conjugated and unconjugated monoclonal antibodies. For each HRP-conjugated monoclonal antibody, a constant concentration of antigen coated to plastic wells was used for the competition experiments. The concentration of CMV antigen (equivalent to 0.2 CF antigen units per ml) coated was sufficient to give an OD value of about 1.000 at the end of the experiment. Nonspecific binding of the labeled antibodies was assayed on wells coated with 5 μg of normal cell protein per ml and was insignificant in all cases.

Dilutions of each monoclonal antibody (2, 20, and 200 μg/ml) were mixed with an equal volume (a fixed concentration) of homologous or heterologous HRP-conjugated antibody in PBS–1% Triton X-100, and 50 μl was applied to CMV antigen- or normal cell antigen-coated wells. The amount of unlabeled competing antibody added to each well was estimated by the ELISA. The results are expressed as percent competition determined by a formula described by Breschkin et al. (4): percent competition = 100 × [1 − (HRP conjugate found in the presence of unlabeled antibody/HRP conjugate bound in the absence of unlabeled antibody)].

Sephadex G-200 column chromatography. CMV CF antigen or purified virus treated with either 1% Triton X-100 (30 min at 37°C) or 4% SDS and 5% mercaptoethanol (2 min boiling) were chromatographed in a column (1.5 by 100 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Stockholm, Sweden). PBS (0.01 M [pH 7.4] containing 0.1% Triton X-100 or 0.1% SDS) was used as eluent for Triton X-100 and SDS-treated preparations, respectively.

RESULTS

Establishment of hybrid clones secreting antibodies reactive with CMV antigen. After fusion of spleen cells from CMV-immunized mice with myeloma cells as described in Materials and Methods, 5×10^4 myeloma cells in 0.1 ml were added to each well in 96-well tissue culture clusters. After 3 to 4 weeks of incubation, hybrid cell growth was detected in 66 of 600 wells. By ELISA the supernatant fluids from 32 of the 66 hybrids were found to contain CMV-specific antibody. The remaining hybrids either produced antibody reactive with normal cell antigen or failed to produce detectable amounts of antibody. The hybrids secreting CMV-specific antibody were cloned three times, and nine stable cloned hybrid lines (1B8, 4D2, 4D11, 7B4, 7D2, 7E2, 8E3, 8E9, and 8E10) were established. These were transferred from the wells into tissue culture flasks for further studies. Eight hybrid clones secreted the IgG1 subclass of immunoglobulin, whereas cloned hybrid line 8E3 secreted the IgG2b subclass of immunoglobulin.

Ascites fluid for each clone was prepared as described in Materials and Methods. The relative antibody titers of tissue culture fluid and ascites fluid were determined by ELISA. A serum pool from three normal mice was used as a negative control. The mean OD value obtained at 1:100 dilutions of normal mouse serum plus 2 standard deviations was used as a cutoff point. The antibody titers for tissue culture fluids were 1:16,384 for 1B8, 4D11, 7B4, and 8E10; 1:1,024 for 7D2, 7E2, 8E3, and 8E9; and 1:256 for 4D2. The antibody titers of ascites fluids were 1:10,485,760 for 4D11, 1B8, and 8E3; 1:2,621,440 and 7B4, 8E9, and 8E10; and 1:650,360 for 7E2, 4D2, and 7D2.

None of the antibodies neutralized CMV at 100, 10, and 1 μg of antibody per ml. A mixture of these nine monoclonal antibodies also failed to exhibit any virus-neutralizing activity.

Determination of antigenic determinants specific for each monoclonal antibody by competitive binding assay. For each monoclonal antibody, a fixed amount of HRP-conjugated antibody (0.15 μg/ml) was mixed with various amounts (2, 20, 200 μg/ml) of either unlabeled homologous or one of the other eight monoclonal antibodies (heterologous) and added to plastic wells coated with a limiting concentration of CMV antigen. Antibodies 8E9, 7E2, and 4D2 effectively competed with each other (Table 1) and therefore appear to be directed against the same epitope or against antigenic determinants located close to each other. Similarly, 7B4 and 8E10 are against the same epitope or against antigenic determi-

TABLE 1. Competitive binding assays using a constant amount of HRP-conjugated monoclonal antibodies with homologous and heterologous unlabeled monoclonal antibodies

Competing unlabeled monoclonal antibody	Maximal competition observed (%) with given HRP-conjugated monoclonal antibodies								
	8E9	7E2	4D2	4D11	7B4	8E10	7D2	8E3	1B8
8E9	99	87	99	0	0	0	0	0	0
7E2	97	88	94	0	0	0	0	0	0
4D2	96	90	95	0	0	0	0	0	0
4D11	0	0	0	98	0	0	0	0	0
7B4	0	0	0	0	94	96	0	0	0
8E10	0	0	0	0	98	98	0	0	0
7D2	0	0	0	0	0	0	92	0	0
8E3	0	0	0	0	0	0	0	96	0
1B8	0	0	0	0	0	0	0	0	98

nants in the same region. With monoclonal antibodies 1B8, 8E3, 4D11, and 7D2, competition was demonstrated only when homologous unlabeled antibody was mixed with HRP-conjugated antibody. These nine monoclonal antibodies were then divided into six groups, each reacting with specific antigenic determinants or a specific region with closely situated antigenic determinants on CMV antigens. These are groups A (4D11), B (7B4, 8E10), C (7D2), D (8E9, 7E2, 4D2), E (8E3), and F (1B8).

Identification of CMV polypeptides reactive with monoclonal antibodies. The immunoblot method (Western blot) was used to identify CMV-specified polypeptides which had specific antigenic reactivity for the nine monoclonal antibodies. Purified CMV (strain AD169) and a glycine-extracted CMV antigen preparation were electroblotted onto a nitrocellulose sheet from a 9% polyacrylamide gel containing 0.1% SDS on which they had been previously run. The electroblotted polypeptides were tested for reactivity with each monoclonal antibody. A polypeptide band with a relative molecular weight of 66,000 present in both purified virus and CMV CF antigen preparations reacted with monoclonal antibodies 4D11, 7B4, 7D2, 8E3, and 8E10. None of the polypeptide bands present in normal F-5000 cells reacted with any of the monoclonal antibodies (Fig. 1 and 2). Normal mouse serum or goat anti-mouse IgG HRP conjugates did not bind to any of the polypeptide bands, including the 66,000-dalton polypeptide. Preincubation of 4D11 antibody with antigen preparations made from HSV-1-, HSV-2-, or varicella-zoster virus-infected cells failed to prevent this antibody from binding to the 66,000-molecular-weight electroblotted polypeptide band. However, 4D11 antibody absorbed with CMV antigen failed to react with the polypeptide band with 66,000 molecular weight or any other bands in the electroblot. The monoclonal antibodies in groups D (8E9, 7E2, 4D2) and F (1B8) did not react with any of the polypeptide bands seen in the electroblot.

Identification of the CMV polypeptides reactive with group D (4D2, 7E2, 8E9) and F (1B8) antibodies by double-antibody sandwich ELISA. Using six monoclonal antibodies (one from each group; 1B8, 8E3, 4D11, 7B4, 7D2, and 8E9) in different combinations, the double-antibody sandwich ELISA technique was performed with the fractions obtained from the Sephadex G-200 column chromatography of purified virus and CF antigen. Fractions from the chromatographed Triton X-100-treated CF antigen with molecular weight range from near the void volume (200,000) to about 25,000 contained antigen reactive with the six monoclonal antibodies (Fig. 3). Reactivity was greatest in the size range from about 160,000 to 60,000 daltons, with three apparent peaks occurring at molecular weights of approximately 160,000, 110,000, and 68,000. Purified CMV treated with 1% Triton X-100 and chromatographed on Sephadex G-200 showed the most reactivity to the monoclonal antibodies in the fractions with a size range from near the void volume (200,000) to 160,000 daltons and a small peak near 68,000 daltons. The above experiment showed that Triton X-100 treatment did not dissociate reactive polypeptide(s) into a discrete molecular weight species for identification. Although only some of the combinations of the six monoclonal antibodies for the detection of the antigens are shown in Fig. 3, any one of the six monoclonal antibodies was equally effective in the capture of antigen and any one of the six HRP-conjugated monoclonal antibodies was equally effective in detecting the captured antigen. In a second set of experiments, SDS (1% final concentration; 2 min at 100°C) was used to solubilize CMV preparations. When CMV CF antigen was treated and then chromatographed on a Sephadex G-200 column, fractions in the molecular weight range of 120,000 showed the most reactivity to the four monoclonal antibod-

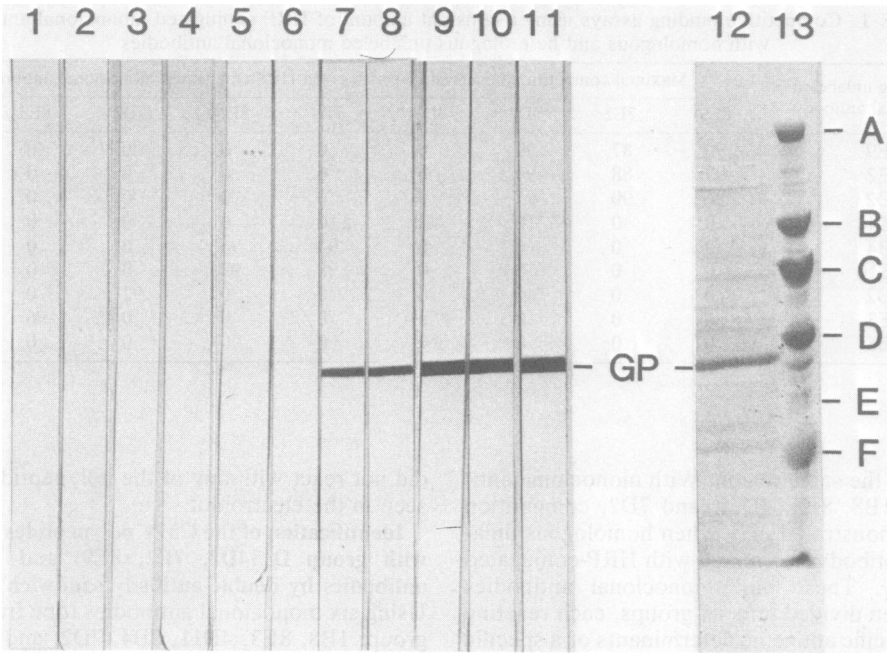

FIG. 1. Immunoblot demonstration of reactivity of nine monoclonal antibodies to polypeptides present in purified CMV. Immunoblots were prepared from an SDS-polyacrylamide gel electrophoresis slab (9%) in which a sample of purified virus was applied and run as a continuous band across the gel except for a small slot on the left and right of the gel for molecular weight markers. The separated polypeptides were electrophoretically transferred to a sheet of nitrocellulose, which was then cut into approximately 1-cm-wide vertical strips; each strip was incubated with one of the monoclonal antibodies followed by incubation with goat HRP-conjugated anti-mouse IgG antibody and reacted with 4-chloro-1-naphthol substrate solution. Electroblot strips were stained with (1) normal mouse serum, (2) goat HRP-conjugated anti-mouse IgG antibody, (3) 1B8, (4) 4D2, (5) 7E2, (6) 8E9, (7) 4D11, (8) 7B4, (9) 7D2, (10) 8E3, and (11) 8E10. Lanes 12 and 13 represent electroblots stained with amido black containing separated CMV polypeptides and molecular weight markers, respectively. GP, Glycosylated polypeptide (GP66). Molecular weight markers are described in the text.

ies (8E3, 4D11, 7B4, and 7D2), with minor peaks at 66,000 and 30,000 daltons (Fig. 4A). Apparently GP66 was not completely solubilized or reassociated with other proteins. Purified CMV treated with SDS in the same manner and chromatographed on Sephadex G-200 showed fractions in the molecular weight range of 66,000 to be the most reactive with the same four monoclonal antibodies. In contrast to Triton X-100-treated preparations, fractions from SDS-treated CMV CF antigen or purified CMV chromatographed on a Sephadex G-200 column showed very little reactivity that was specific for the 1B8 and 8E9 antibodies (Fig. 4B). However, the reactivity which did remain was all at the 66,000-molecular-weight region. This reactivity was detected by using either of these antibodies with 4D11, 7B4, 7D2, or 8E3 antibodies in the double-antibody sandwich ELISA. Only some of the combinations of the double-antibody sandwich ELISA technique used for detection of the antigen are shown in Fig. 4A and B. When the antigen titer was determined before and after treatment with SDS by the double-antibody sandwich ELISA, using 4D11, 7B4, 8E3, and 7D2, it did not decrease (data not shown).

Determination of optimum combinations for the capture and detection of CMV antigen, using six monoclonal antibodies for the double-antibody sandwich ELISA. All possible pair combinations from the six groups of monoclonal antibodies were used as either the capture antibody or the detector antibody (HRP-conjugated antibody) to find the optimum combination for the detection of antigen. Serial dilutions of a CMV CF antigen preparation with a known number of CF antigen units were assayed. The CMV CF antigen preparation was solubilized with Triton X-100 (1% final concentration) for 30 min at 37°C in a water bath. Twofold serial dilutions of the supernatant fluid were made, and these were assayed by the double-antibody sandwich ELISA to determine the minimum amount of CF antigen detectable by this method. All combinations detected a

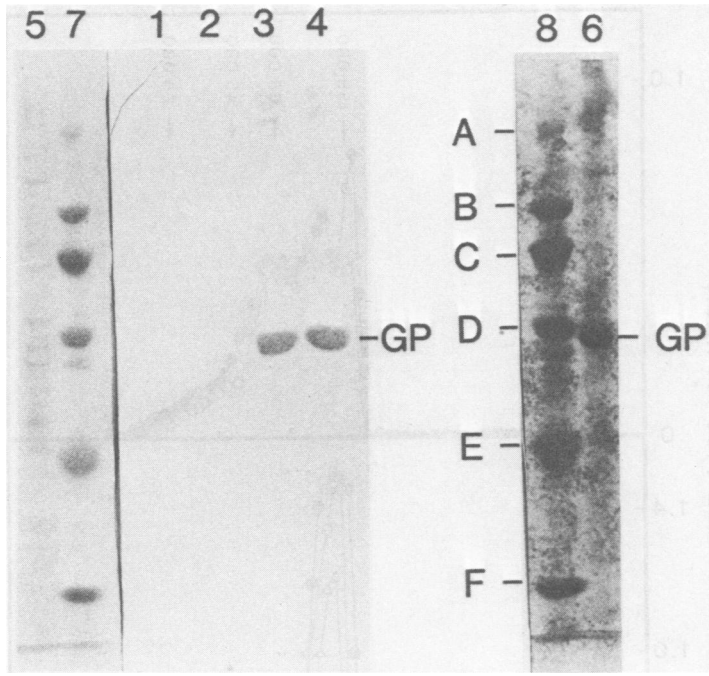

FIG. 2. Immunoblot demonstration of reactivity of 4D11 monoclonal antibody to polypeptides present in CMV CF antigen preparations. Immunoblots were prepared from an SDS-polyacrylamide gel electrophoresis slab (9%) on which the SDS-solubilized CMV antigen and control normal F-5000 cell antigen had been electrophoresed. Immunoblots were prepared as described in the legend to Fig. 1. The electroblotted nitrocellulose sheet was divided vertically into three sections. Section 1 containing lanes 1 and 2 (normal control cell CF antigen preparation) and 3 and 4 (CMV CF antigen preparation) were immunoblotted with 4D11 monoclonal antibody. Section 2 containing lanes 5 (normal control CF antigen preparation) and 7 (molecular weight markers) and section 3 containing lanes 6 (CMV antigen preparation) and 8 (molecular weight markers) were stained with amido black. GP, Glycosylated polypeptide (GP66). Molecular weight markers are described in the text.

small amount of CMV CF antigen (Table 2). When one monoclonal antibody was used for both capture antibody and detector antibody, the sensitivity of the test was lower. Pair combinations of monoclonal antibodies, each specific for different epitopes, were consistently more sensitive and could detect as little as 0.000245 U of CMV CF antigen. The same sensitivity was obtained by using a monoclonal antibody (4D11) as a capture antibody and a mixture of three HRP-conjugated monoclonal antibodies (7B4, 7D2, and 8E9), each reacting with different epitopes, as the detector antibody.

Estimation of GP66 in CMV-infected cells and its culture fluid by the double-antibody sandwich ELISA. CMV-infected cells (multiplicity of infection, 3 PFU) showing 100% cytopathic effect at 6 days p.i. were used, and infected cells in culture fluid as well as infected culture fluid (after the infected cells were removed by centrifugation at 2,000 × *g* for 10 min) were tested. Two monoclonal antibodies that react with different epitopes (4D11 antibody and HRP-conjugated 7B4 antibody) were used. As little as 50% of GP66 present in one infected cell per well could be detected (Table 3). The relative concentration of GP66 in infected cells was 16-fold higher than that found in infected culture fluid.

Detection of CMV antigen (GP66) present in F-5000 cells infected with different laboratory strains and clinical isolates of CMV by double-antibody sandwich ELISA. Tissue culture test tubes containing 1.2×10^5 F-5000 cells infected with various strains of CMV exhibiting at least 25% cytopathic effect were solubilized with 1 ml of PBS containing 1% Triton X-100 for 30 min at 37°C and tested for the presence of GP66 by the double-antibody sandwich ELISA, using 4D11 and HRP-conjugated 8E9 monoclonal antibody as capture and detector antibodies, respectively. Five clinical isolates as well as six laboratory strains of CMV (Davis, Espilat, Kerr, Towne, C-87, and G-35) gave positive results for the presence of GP66 antigen (Table 4). When F-5000 cells infected with HSV-1, HSV-2, and varicella-zoster virus exhibiting 100% cytopath-

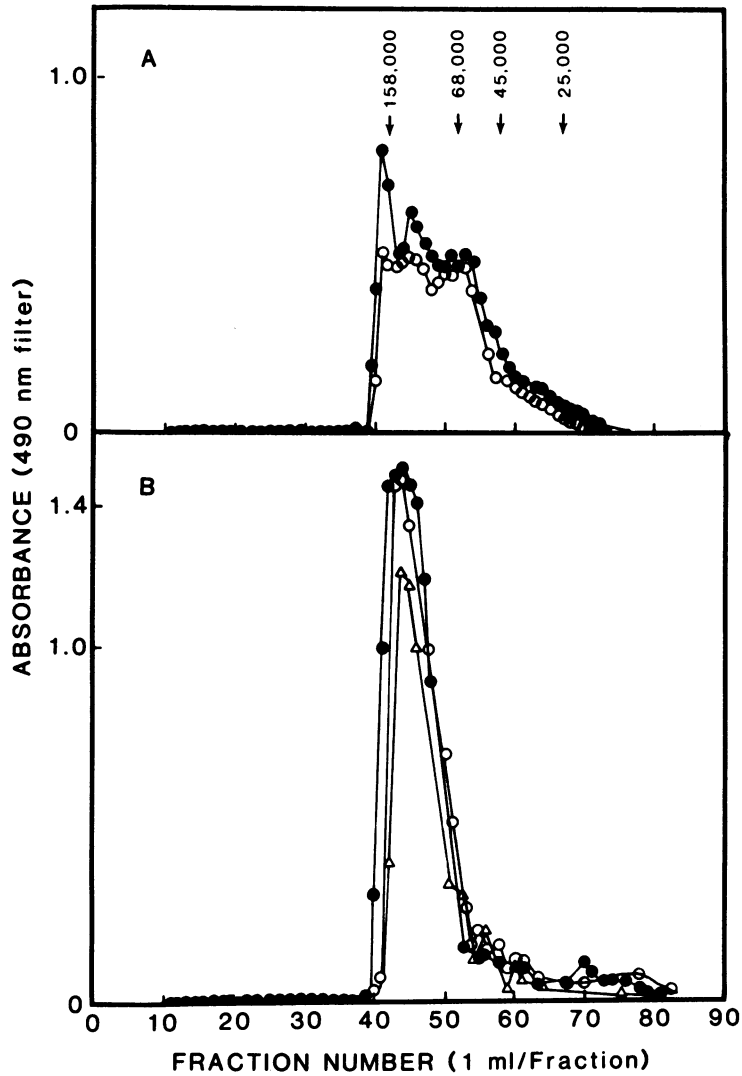

FIG. 3. Sephadex G-200 column chromatography of (A) Triton X-100-treated CMV antigen preparation and (B) Triton X-100-treated purified CMV. Absorbance = OD value of color reaction read at 490 nm for HRP-conjugated monoclonal antibodies specific to CMV antigen, using the double-antibody sandwich ELISA technique with OPD as the HRP substrate. Symbols: (A) ●, 8E9 (capture) antibody + fractionated sample + HRP-conjugated 4D11 (detector) antibody; ○, 4D11 (capture) antibody + fractionated sample + HRP-conjugated 8E9 (detector) antibody. (B) ●, 4D11, (capture) antibody + fractionated sample + HRP-conjugated 8E9 (detector) antibody; ○, 8E9 (capture) antibody + fractionated sample + HRP-conjugated 4D11 (detector) antibody; △, 4D11 (capture) antibody + fractionated sample + HRP-conjugated 7B4 (detector) antibody. Molecular weight standards are as follows: 158,000 (aldolase), 68,000 (bovine serum albumin), 45,000 (ovalbumin), and 25,000 (chymotrypsinogen A). Arrows indicate what fraction number corresponds to stated molecular weight.

ic effect or Epstein-Barr virus antigen (Epstein-Barr nuclear antigen CF titer, 4 U) were tested, none gave positive reactions. The same result was obtained when HRP-conjugated 8E9, 7D2, 1B8, or 8E3 was used as detector antibody (data not shown).

Detection of GP66 synthesized in CMV-infected cells at different times p.i. Monolayers containing approximately 1.5×10^6 cells were infected with CMV (strain AD169) at a multiplicity of infection of 3 PFU. After 2 h of virus adsorption at 37°C, cells were washed three times with growth medium and then growth medium was added. At different times p.i. the amount of antigen in both the supernatant fluid and solubilized cells (1% Triton X-100) was tested, using the double-

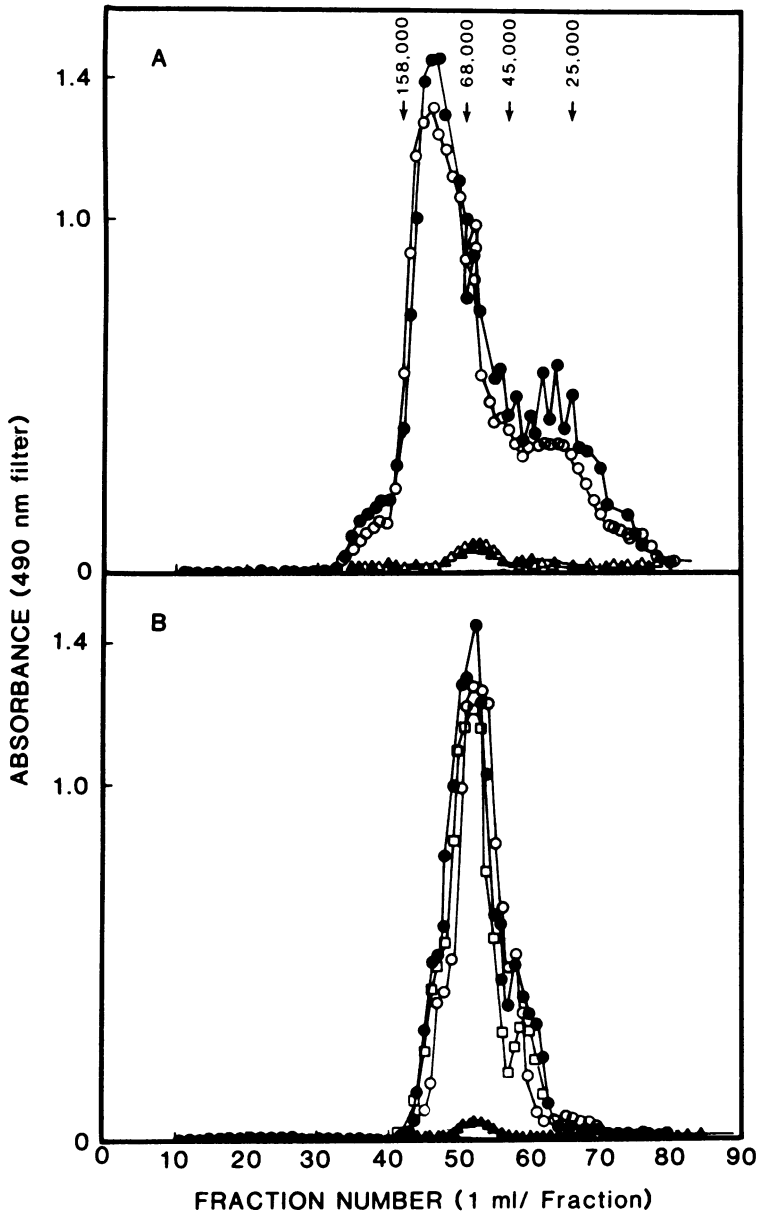

FIG. 4. Sephadex G-200 column chromatography of SDS-treated (A) CMV CF antigen preparation and (B) CMV. Absorbance = OD value of color reaction read at 490 nm for HRP-conjugated monoclonal antibodies specific to CMV antigen, using the double-antibody sandwich ELISA technique with OPD as the HRP substrate. Symbols: ●, 4D11(capture) antibody + fractionated sample + HRP-conjugated 7B4 (detector) antibody; ○, 4D11 (capture) antibody + fractionated sample + HRP-conjugated 7D2 (detector) antibody. ▲, 4D11, (capture) antibody + fractionated sample + HRP-conjugated 8E9 (detector) antibody; △, 8E9 (capture) antibody + fractionated sample + HRP-conjugated 4D11 (detector) antibody; □, 7B4 (capture) antibody + fractionated sample + HRP-conjugated 4D11 (detector) antibody. See legend to Fig. 3 for molecular weight standards. Arrows indicate what fraction number corresponds to stated molecular weight.

antibody ELISA with antibody 4D11 as the capture antibody and HRP-conjugated 7B4 antibody as the detector antibody. GP66 was detected in the solubilized infected cells starting from 2 h p.i. Detectable amounts of GP66 were also found in the supernatant fluid at 2 h p.i. The amount of this specific glycosylated polypeptide detectable by the ELISA increased with time.

TABLE 2. Determination of optimum combination of capture antibody and detector antibody for CMV antigen, using six monoclonal antibodies for the double-antibody sandwich ELISA test

Capture antibody	Amt of CF antigen detectable (U/50 μl)	Detector antibody (HRP conjugated)
8E9	0.039	8E9
8E9	0.000245	4D11
8E9	0.000245	7B4
8E9	0.000490	7D2
8E9	0.000490	1B8
8E9	0.000490	8E3
4D11	0.00195	4D11
4D11	0.000245	7B4
4D11	0.000245	7D2
4D11	0.000245	8E9
4D11	0.000245	1B8
4D11	0.000245	8E3
7B4	0.0039	7B4
7B4	0.000245	4D11
7B4	0.000245	7D2
7B4	0.000245	8E9
7B4	0.000490	1B8
7B4	0.000245	8E3
7D2	0.00195	7D2
7D2	0.000490	4D11
7D2	0.000490	7B4
7D2	0.000490	8E9
7D2	0.000490	1B8
7D2	0.000490	8E3
1B8	0.000490	8E9
1B8	0.000490	4D11
1B8	0.000245	7B4
1B8	0.000490	7D2
1B8	0.039	1B8
1B8	0.000495	8E3
8E3	0.000245	8E9
8E3	0.000245	4D11
8E3	0.000245	7B4
8E3	0.000490	7D2
8E3	0.000490	1B8
8E3	0.00195	8E3

IFA of CMV-infected cells. Monoclonal antibodies (4D11, 7B4, 7D2, 8E9, 8E3, and 1B8) were tested for their ability to react with CMV antigen present in CMV-infected F-5000 cells at 24 p.i. by the IFA. Although all six monoclonal antibodies reacted with antigen present primarily in the nuclei of the infected cells, 4D11 and 8E3 gave the strongest fluorescent reactions. By 48 and 72 h p.i. monoclonal antibodies reacted strongly with antigen present in both the cytoplasm and the nuclei of infected cells. An intense immunofluorescent reaction from many various-sized granules was typically seen in infected cells stained with these monoclonal antibodies. Kinetic studies demonstrated faint but detectable amounts of fluorescence in nuclei of CMV-infected cells at 2 h p.i. when monoclonal antibody 4D11 or 8E3 was used. Thereafter the amount of antigen increased so that CMV-infected cells at 6 h p.i showed highly intense fluorescence. Control uninfected F-5000 cells were negative by the IFA. F-5000 cells infected with six other strains of CMV (Davis, Kerr, Espilat, C-87, Towne, and G-35) and five clinical isolates of CMV gave positive results when reacted with 8E3 or 4D11 antibody, and they were indistinguishable from F-5000 cells infected with CMV strain AD169. Monoclonal antibodies 4D11 and 8E3 did not react with F-5000 cells infected with HSV-1, HSV-2, or varicella-zoster virus in the same IFA test. Monoclonal antibodies obtained from tissue culture supernatant fluids and ascites fluid were both effective in the IFA technique.

DISCUSSION

The primary purposes of this study were to develop a panel of monoclonal antibodies against a major glycosylated polypeptide of CMV (GP66) which could be used to develop a highly sensitive and objective double-antibody sandwich ELISA to detect GP66 and then to eventually use this method to detect the presence of CMV in clinical samples. GP66 has been identified as the major glycosylated polypeptide of CMV by Kim et al. (14). We successfully developed nine hybrid cell lines (1B8, 4D2, 4D11, 7B4, 7D2, 7E2, 8E3, 8E9, and 8E10) which secrete antibody that react with CMV-specific antigen. By the competitive binding inhibition assay (4), using fixed amounts of HRP-conjugated antibody with various concentrations of unlabeled homologous and heterologous antibody on fixed amounts of CMV antigen coated onto the test wells, the nine clones were divided into six groups, each reacting with a different epitope or region containing antigenic determinants so closely located as to cause binding interference. They are groups A (4D11), B (7B4, 8E10), C (7E2), D (4D2, 7E2, and 8E9), E (8E3), and F (1B8). The extent of binding competition observed among antibodies within each group was equal so that the distinction of antibodies within each group was not possible. Significantly, the four distinct regions of antigenic determinants corresponding to group A, B, C, and E monoclonal antibodies on GP66 appeared to be quite stable in that SDS treatment failed to denature the antigenic determinants, and they probably represent regions of antigenic determinants on the primary structure of GP66.

Therefore, a test detecting such stable anti-

TABLE 3. Double-antibody sandwich ELISA for measurements of GP66 present in CMV-infected cells and infected cell culture fluid[a]

Dilution	No. of infected cells per 50 μl of culture fluid	OD value	OD value obtained from equivalent normal F-5000 cells in 50 μl	OD value of infected cell culture fluid[b]
Undiluted	1,224	6.440	0.001	2.301
1:2	512	5.920	0.003	1.562
1:4	256	5.600	0.006	1.165
1:8	128	4.568	0.008	0.490
1:16	64	2.551	0.003	0.233
1:32	32	2.103	0.005	0.079
1:64	16	1.189	0.002	0.034
1:128	8	0.663	0.006	0.021[c]
1:256	4	0.516	0.001	0.011
1:512	2	0.156	0.002	0.009
1:1,024	1	0.070	0.004	0.002
1:2,048	0.5	0.028[b]	0.002	0.011
1:4,096	0.25	0.013	0.003	0.006
1:8,192	0.125	0.008	0.004	0.003

[a] Mean ($\bar{x}$) OD value for normal cell column = 0.0036. Standard deviation (SD) = 0.0057. Cutoff point for positive OD value = $\bar{x}$ + 2SD = 0.0036 + (2 × 0.0057) = 0.015.

[b] OD value of infected cell culture fluid after removal by centrifugation of infected cells for 10 min at 120 × *g*.

[c] Endpoint for antigen presence.

genic determinants on GP66 should provide a highly reliable method for detecting the presence of CMV in clinical specimens. Further studies with the double-antibody sandwich ELISA indicated that the regions of antigenic determinants specific for group A, B, C, and E monoclonal antibodies were not destroyed at all by SDS treatment. Group D and F monoclonal antibodies appeared to be reactive to CMV antigenic determinants, which are readily denatured by SDS treatment. As demonstrated by immunoblots, none of the SDS-denatured polypeptide bands reacted with group D and F monoclonal antibodies. It was further demonstrated that fractions from both SDS-treated purified CMV and CMV CF antigen chromatographed on a Sephadex G-200 column lost most of their reactivity to group D and F monoclonal antibodies. However, it is interesting to note that the double-antibody sandwich ELISA that used group D and F antibody as either capture or detector antibody in combination with any one of the A, B, C, or E group monoclonal antibodies was able to detect very small amounts of CMV antigen in SDS-treated CMV or CMV CF antigen. These findings, plus the fact that the fractions near the molecular weight region of 66,000 were the only antigenic peak detected when SDS-treated preparations were used, appear to suggest that group D and F antibodies are probably also reactive to GP66. This may be due to the presence of a small amount of the antigenic determinant specific for group D or F antibody withstanding denaturation by the SDS or to some degree of renaturation after SDS treatment. However, further studies are needed to definitely identify CMV polypeptides reactive with group D and F monoclonal antibodies.

When Sephadex G-200 column chromatography was performed on Triton X-100-treated purified CMV and CMV CF antigen and each

TABLE 4. Detection of CMV antigen (GP66) present in F-5000 cells infected with laboratory strains and clinical isolates of CMV, using 4D11 monoclonal antibody bound to the solid phase for capturing GP66 antigen and HRP-conjugated 7B4 monoclonal antibody for detection of the captured GP66 antigen[a]

CMV strain	Avg OD value of 4 tests (E490)
AD169	0.263
Esp.	0.192
Kerr	0.425
Davis	0.327
G-87	0.492
G-35	0.568
Towne	0.425
Primary isolates	
A-106	0.420
A-148	0.235
A-164	0.415
HD	0.321
DH	0.412
HSV-1	0.004
HSV-2	0.003
EBNA CF antigen[b]	0.002
Normal F-5000 cell antigen	0.003

[a] Cutoff between positive and negative OD value = mean of normal F-5000 cell antigen OD value (0.004) + 2 standard deviations (2 × 0.002) = 0.003 + 0.004 = 0.008.

[b] EBNA, Epstein-Barr virus nuclear antigen.

fraction was tested by the double-antibody sandwich ELISA, using all possible pair combinations of monoclonal antibodies, GP66 was distributed in the molecular weight range from near the void volume, 200,000, to <60,000, suggesting that GP66 is associated with different-sized proteins. Furthermore, Triton X-100 treatment did not solubilize GP66. As long as the antigen was treated with Triton X-100, group D and F monoclonal antibodies were equally effective in capturing and detecting CMV antigen.

That group D and F antibodies did react slightly with the SDS-treated material in the double-antibody ELISA but did not react with the GP66 band on electroblots may be due to the greater sensitivity of the double-antibody sandwich ELISA technique, which uses the soluble substrate OPD. Further characterization by the immune precipitation technique described by Periera et al. and Goldstein et al. (8, 16) is currently in progress for these monoclonal antibodies.

By use of the ELISA the amount of GP66 synthesized and accumulated in CMV-infected cells at different times after infection was determined. In the very early phase of CMV replication (0 to 2 h p.i.) GP66 was synthesized in a sufficient amount to be detected by the ELISA. Thereafter, the amount of GP66 synthesized increased rapidly, and by 8 h p.i. a strong positive reaction was recorded with the double-antibody sandwich ELISA.

By polyacrylamide gel electrophoretic analysis a polypeptide with the same molecular weight was detected in CF antigen preparations of other laboratory strains of CMV (Towne, Espilat, Davis, Kerr, and C87). This is probably a glycoprotein similar if not identical to GP66 which is common in many, if not all, strains of CMV. This conclusion is firmly supported by the fact that, by using our monoclonal antibodies 4D11 and 7B4 in the double-antibody sandwich ELISA, five clinical isolates and other laboratory strains of CMV gave unequivocal positive results. GP66 appears to be unique to CMV in that HSV-1, HSV-2, varicella-zoster virus, or Epstein-Barr virus nuclear antigen gave negative results by the ELISA, indicating a complete lack of cross-reactivity.

Preliminary data also indicate that in CMV-infected cells stained by the IFA method, using monoclonal antibodies 4D11 and 8E3, CMV antigen can be detected as early as 2 h p.i. GP66 has been shown to be a CMV viral structural polypeptide by Kim et al. (14). Recently, it has been demonstrated that a glycosylated polypeptide with molecular weight of 68,000 is also an immediate early protein synthesized in CMV-infected cells (24). The major glycosylated polypeptide detected in our laboratory is consistently 66,000 molecular weight (13, 14). Early detection of GP66 by IFA, using 4D11 antibody and double-antibody sandwich ELISA reported here, confirmed the fact that GP66 is one of the immediate early proteins.

The utilization of these monoclonal antibodies which are reactive to GP66 seem to provide a powerful immunological tool for detection of CMV. In humans, antibody response to this glycosylated polypeptide (immediate early protein) is very pronounced. Antibody to GP66 is detected early in acute sera from CMV-infected patients (17). The antibody was present in children with perinatal and congenital infections.

A highly sensitive double-antibody sandwich ELISA for the detection of antigen was devised, using one monoclonal antibody (4D11) as the capture antibody and the second monoclonal antibody (7B4), which reacts with a different antigenic determinant on the same glycoprotein, as the detector antibody. This test, which is rapid and objective, could detect as little as 50% of the CMV GP66 present in one CMV-infected cell or as few as 0.000245 CF units of antigen. When an attempt was made to increase the sensitivity of the double-antibody sandwich ELISA by using a mixture of three HRP-conjugated antibodies instead of just one, the sensitivity proved to be the same. An advantage of this assay is that infectious CMV need not be present in the specimen.

For the double-antibody sandwich ELISA to be useful for CMV diagnosis, detection of GP66 must prove to be equal to or greater in sensitivity than the standard clinical procedure for diagnosing CMV infections by isolation of infectious virus. Such comparative studies are now in progress.

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LITERATURE CITED

1. **Alford, C. A., Jr., P. D. Griffiths, S. Stagno, R. F. Pass, and R. J. Smith.** 1982. Congenital cytomegalovirus infection: diagnostic and prognostic significance of the detection of specific immunoglobulin M antibodies in cord serum. Pediatrics **69**:544–549.
2. **Alford, C. A., Jr., S. Stagno, and D. W. Reynolds.** 1975. Diagnosis of chronic perinatal infections. Am. J. Dis. Child. **129**:455–463.
3. **Betts, R. F., R. B. Freeman, R. G. Douglas, Jr., and T. E. Talley.** 1977. Clinical manifestations of renal allograft derived primary cytomegalovirus infection. Am. J. Dis. Child. **131**:759–763.
4. **Breschkin, A. M., J. Ahern, and D. O. White.** 1981. Antigenic determinants of influenza virus hemagglutinin. VIII. Topography of the antigenic regions of influenza virus hemagglutinin determined by competitive radioimmunoassay with monoclonal antibodies. Virology **113**:130–140.
5. **Burnette, W. N.** 1981. "Western blotting": electrophoret-

ic transfer of proteins from sodium dodecyl sulfate-polyacrylamide *gels* to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.

6. **Drew, W. L., R. C. Miner, J. L. Ziegler, J. H. Gullett, D. I. Abrams, M. A. Conant, E.-S. Huang, J. R. Groundwater, P. Volberding, and L. Mintz.** 1982. Cytomegalovirus and Kaposi's sarcoma in young homosexual men. Lancet **ii**:125–127.
7. **Drew, W. L., L. Mintz, R. C. Miner, M. Sands, and B. Ketterer.** 1981. Prevalence of cytomegalovirus infection in homosexual men. J. Infect. Dis. **143**:188–192.
8. **Goldstein, L. C., J. McDougall, R. Hackman, J. D. Meyers, E. D. Thomas, and R. C. Nowinski.** 1982. Monoclonal antibodies to cytomegalovirus: rapid identification of clinical isolates and preliminary use in diagnosis of cytomegalovirus pneumonia. Infect. Immun. **38**:273–281.
9. **Greenberg, H., V. McAuliffe, J. Valdosuso, R. Wyatt, J. Flores, A. Kalica, Y. Hoshino, and N. Singh.** 1983. Serological analysis of the subgroup protein of rotavirus using monoclonal antibodies. Infect. Immun. **39**:91–99.
10. **Hanshaw, J. B.** 1971. Congenital cytomegalovirus infection: a fifteen year perspective. J. Infect. Dis. **123**:555–561.
11. **Hanshaw, J. B., and M. E. Melish.** 1973. Congenital cytomegalovirus infection. Am. J. Dis. Child. **126**:190–194.
12. **Hanshaw, J. B., A. P. Scheiner, A. W. Moxley, L. Gaev, V. Abel, and B. Scheiner.** 1976. School failure and deafness after "silent" congenital cytomegalovirus infection. N. Engl. J. Med. **295**:468–470.
13. **Kim, K. S., H. M. Moon, V. J. Sapienza, and R. I. Carp.** 1977. Complement-fixing antigen of human cytomegalovirus. J. Infect. Dis. **135**:281–288.
14. **Kim, K. S., V. J. Sapienza, R. I. Carp, and H. M. Moon.** 1976. Analysis of structural polypeptides of purified human cytomegalovirus. J. Virol. **20**:604–611.
15. **Pereira, L., M. Hoffman, and N. Cremer.** 1982. Electrophoretic analysis of polypeptides immune precipitated from cytomegalovirus-infected cell extracts by human sera. Infect. Immun. **36**:933–942.
16. **Pereira, L., M. Hoffman, D. Gallo, and N. Cremer.** 1982. Monoclonal antibodies to human cytomegalovirus: three surface membrane proteins with unique immunological and electrophoretic properties specify cross-reactive determinants. Infect. Immun. **36**:924–932.
17. **Pereira, L., S. Stagno, M. Hoffman, and J. E. Volanakis.** 1983. Cytomegalovirus-infected cell polypeptides immune-precipitated by sera from children with congenital and perinatal infections. Infect. Immun. **39**:100–108.
18. **Prince, A. M., W. Szmuness, S. J. Millian, and D. S. David.** 1971. A serologic study of cytomegalovirus infections associated with blood transfusions. N. Engl. J. Med. **284**:1125–1131.
19. **Reynolds, D. W., S. Stagno, K. G. Stubbs, A. J. Dahle, M. M. Livingston, S. S. Saxon, and C. A. Alford.** 1974. Inapparent congenital cytomegalovirus infection with elevated cord IgM levels. N. Engl. J. Med. **290**:291–296.
20. **Sarov, I., and I. Abady.** 1975. The morphogenesis of human cytomegalovirus: isolation and polypeptide characterization of cytomegalovirions and dense bodies. Virology **66**:464–473.
21. **Stagno, S., D. W. Reynolds, C. S. Amos, A. J. Dahle, F. P. McCollister, I. Mohindra, R. Ermocilla, and C. A. Alford.** 1977. Auditory and visual defects resulting from symptomatic and subclinical congenital cytomegaloviral and toxoplasma infections. Pediatrics **59**:669–678.
22. **Starr, J. G., R. D. Bart, Jr., and E. Gold.** 1970. Inapparent congenital cytomegalovirus infection. N. Engl. J. Med. **282**:1075–1078.
23. **Stern, H., and S. M. Tucker.** 1973. Prospective study of cytomegalovirus infection in pregnancy. Br. Med. J. **2**:268–270.
24. **Stinski, M. F.** 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26**:686–701.
25. **Togashi, T., C. Orvell, F. Vartdal, and E. Norrby.** 1981. Production of antibodies against measles virions by use of the mouse hybridoma technique. Arch. Virol. **67**:149–157.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
27. **Waner, J. L.** 1975. Partial characterization of a soluble antigen preparation from cells infected with human cytomegalovirus: properties of antisera prepared to the antigen. J. Immunol. **114**:1454–1457.
28. **Waner, J. L., T. H. Weller, and S. V. Kevy.** 1973. Patterns of cytomegaloviral complement-fixing antibody activity: a longitudinal study of blood donors. J. Infect. Dis. **127**:538–543.
29. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecylsulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
30. **Wentworth, B. B., and L. French.** 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. **135**:253–258.
31. **Wilson, M. B., and P. K. Nakane.** 1978. Developments in the periodate method of conjugating horseradish peroxidase to antibodies, p. 215–225. *In* W. Knapp, K. Holubar, and G. Wick (ed.), Immunofluorescence and related techniques. Elsevier Scientific Publishing Co., Amsterdam.